REMARKS

Applicant acknowledges with appreciation the indication in the Advisory Action that the §102 and §103 rejections based on U.S. Patent No. 5,872,259 and WO 97/32644 have been withdrawn.

The rejection of claims 1-10 under 35 U.S.C. §103(a) as unpatentable over the Davey et al article in view of U.S. Patent No. 4,010,142 to Hurlock et al has been maintained for the reasons given in the attachment to the Advisory Action. The following arguments and attached documents are directed to the comments set forth in the attachment to the Advisory Action.

It appears to be the Examiner's position that the purification process described in Hurlock et al '142 which involves crystallization and recycling of the mother liquor, would be applicable to any type of purification by selective crystallization as it allegedly would not be affected by the specifics of the method. The Examiner concludes that it would have been obvious to one of ordinary skill in the art at the time of the presently claimed invention "to apply the wash and recycle step of Hurlock et al on any purification process using selective crystallization, including those which use emulsion crystallization such as Davey et al." Respectfully, Applicant disagrees.

The basic assumption in the rejection is that selective crystallization for purification is taught by Hurlock et al for achieving separation and purification. This represents an oversimplification of the process of Hurlock et al. The basic problem to be solved in the Hurlock et al reference is the separation of acrylamide from impurities, especially the predominant "trienamide" (heptoanoamide-1,3,5-triene), see column 1, formulae around line 50 and text in lines 61 to 65. The reference

discloses a process of vacuum-stripping at 25-50°C an originally dilute aqueous acrylamide solution to generate an acrylamide concentration of 80%. Thus, most of the mother liquor has been removed and most of the organic compounds present in the dilute aqueous acrylamide solution also will be removed during this distillation process of vacuum-stripping, which basically corresponds to a vacuum steam distillation, as will be recognized immediately by a person skilled in the art.

Those skilled in the art will also recognize that the process in Hurlock et al in fact, involves two processes: (1) purification of acrylamide solution by removal of the trienamide via steam distillation, and (2) crystallization of the acrylamide from the mother liquor to obtain it in solid form and (as this has been removed by the steam distillation) with less of the triene impurity, followed by repetition of this process using only the mother liquor and the wash water (not the condensate) from the preceding acrylamide crystallization. This is evident from Table I and Example 1 in Hurlock et al. In Example 1, a steam distillation first takes place; the vacuum used for evaporation of the acrylamide solution in the first step is adjusted so as not to exceed 50°C (see column 3, lines 65 and 66). When an acrylamide concentration of approximately 80% is reached in the solution due to removal of the solvent, the stripping is stopped.

That, in fact, the trienamide impurity is removed by the steam distillation under the conditions outlined in Hurlock et al can be deduced by a person having ordinary skill in the art from the information in Hurlock et al is evident from the following translation from Römpp Chemielexikon, Georg Thieme Verlag, Stuttgard, 9th ed., Issue 6, page 5000 (see also literature cited therein, copy of the German original is enclosed):

> Steam distillation is a frequently used technique to extract organic compounds, which have a high boiling point and a low solubility in water. These high boiling compounds can be distilled already at a temperature around 100°C (at 1013 mbar), either if they are boiled together with water or a water steam is led through the mixture. To the low vapour pressure of the organic component the high vapour pressure of the water steam is added (Daltons rule) to reach easily a sum of 1013 mbar (or a lower value if performed under reduced pressure), to that (a lot of) water and (a little bit of) the organic compound are distilled off at the same time.

This teaching can be generalized as follows: As this steam distillation is based on a generally valid physical principle, it can also be performed under reduced pressure and accordingly reduced temperature (vacuum steam distillation). A recent example herefore is the vacuum steam distillation used by Pennarun et al. (copy enclosed) to extract organic components from raw oysters at 25°C.

The polarity of the triene with its longer carbon chain, when compared with acrylamide itself, is much lower than that of acrylamide. Therefore, the miscibility of the triene with the water used as solvent in Hurlock et al is low, while that of acrylamide is quite high (80% concentration at 50°C is possible, as evidenced in Hurlock et al), and thus the triene can be readily removed with evaporating water. Due to its lower solubility, it is thus preferentially removed by the stripping process and finally discarded with the <u>condensate</u> resulting from the stripping process (which condensate is <u>not recycled</u> in Hurlock et al, as this would logically re-introduce the impurity).

To provide evidence of this removal of the triene by steam distillation, two references are enclosed which show comparable processes for comparable compounds: In A.-L. Pennarun et al., *J. of the Science of Food Culture* 82(14), 1652-1660 (2002), compounds contributing to the aroma of oysters are extracted by

vacuum steam distillation at 20°C (see introductory abstract of the enclosed copy of the article). Among the compounds thus distilled from the aqueous oyster medium are various medium-chain aldehydes and alcohols, such as 1-octen-3-ol, nonanal, pentenal, pentenol, hexanal, heptadienol and the like (see page 1655, left column first paragraph). Also, for example, pentanoic acid, octanoic acid, heptanol, 2-butoxyethan-1-ol, 3-methylthio-1-propanol or the like which have comparable molecular structure to that of the triene impurity in Hurlock et al (see Table 1 of Pennarun et al). This is evidence that this type of vacuum steam distillation is applicable for removal of the triene in Hurlock et al.

Another article by Pfeifhofer, W.W. in Flavour and Fragrance Journal 15, 266-270 (2000) provides a further example of such a process. There, the volatile metabolites of Pinus canariensis are obtained by hydrodistillation of pine needles cut into small pieces. A number of monoterpenes (with 10 carbon atoms) and also 2-hexenal are found in the condensate (see paragraph bridging the left and right column on page 267).

Mechanistically, in Hurlock et al the evaporation is far more than a mere concentration of acrylamide for subsequent crystallization. The evaporation step also appears to contribute essentially to the removal of the undesired triene. If significant purification is attained during the crystallization step, then the residual mother liquor will be enriched in the trieneamide. Since, in the subsequent steam distillation step, the concentration of the trienamide is increased, a larger portion of the trienamide will be removed during such subsequent distillation. Thus, the steam distillation process also appears to be a critical purification process.

Applicant reiterates that the evaporated liquid in the Hurlock et al process is

not recycled – which logically follows otherwise the impurity would be re-introduced

into the mother liquor. Only the mother liquor and the washing liquid, which is used

for washing the acrylamide crystals, are recycled. Thus, there is actually no

complete recycling of the water used as solvent. Instead, additional fresh diluted

acrylamide solution is added to allow for the repetition of the vacuum steam

distillation. There is no recycling of the mother liquor as is claimed in the current

patent application, since the main part of the original solution, i.e., the condensate, is

in fact discarded. Therefore, the Hurlock et al process clearly does not correspond

to the process claimed in the present application.

On the other hand, Davey et al is directed solely to emulsion crystallization.

There is no mention or suggestion therein of recycling. Actual crystallization is

employed to obtain pure product crystals. To use the emulsion

crystallization/vacuum steam distillation process disclosed in Hurlock et al in the

process of Davey et al is not suggested in the references nor is there any motivation

for a person skilled in the art to combined emulsion crystallization and recycling.

Moreover, applying a steam distillation step as in Hurlock et al with enrichment of the

desired compound to an emulsion will most certainly lead to a breakdown of the

emulsion as would be recognized by those of ordinary skill. Thus, there would have

been no motivation to combine Hurlock et al and Davey et al.

This conclusion is further supported by the disclosure in Davey et al in the last

paragraph on page 666, which suggests a totally different strategy for further

improvement of their enrichment of m-chloro-nitrobenzene from mixtures with

undesired p-chloro-nitrobenzene. In that paragraph, they suggest as an alternative,

adding specific crystallization inhibitors for the undesired compound. Recycling is not mentioned as an alternative. From this perspective, there would have been no motivation whatsoever to combine the Hurlock et al and the Davey et al references to arrive at the present invention which offers a totally new principle that is not prone to the limitations of Hurlock et al (mandatory inclusion of a steam distillation process) and Davey et al alternative (suggestion of using crystallization inhibitors rather than recycling).
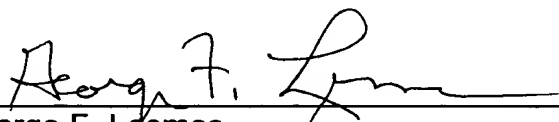
For at least the reasons enumerated above, the combined disclosures of Davey et al and Hurlock et al '142 fail to render obvious the process described in claims 1-10. Accordingly, the §103(a) rejection based on combining the above-mentioned documents should be withdrawn and such action is earnestly solicited.

From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order and such action is earnestly solicited. If there are any questions concerning this paper or the application in general, the Examiner is invited to telephone the undersigned at (703) 838-6683 at his earliest convenience.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: September 20, 2004

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Zentralredaktion: Dr. Elisabeth Hillen

Mit einem Nachwort der Herausgeber

Gesamtregister aus den Bänden 1–6:
– Summenformeln
– Übersetzungen in vier Sprachen

Identification and origin of the character-impact compounds of raw oyster *Crassostrea gigas*

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Abstract: The French consume large amounts of raw oysters. The study of the aroma of oyster *Crassostrea gigas* is of economic interest because it is a good method of checking the sensory quality. Aromas were extracted by vacuum steam distillation at 20°C using whole oyster flesh. This extract presented similar sensory characteristics to raw oyster. The odour-active compounds were characterised by gas chromatography coupled with olfactometry using a panel of 10 judges trained in seafood aroma recognition. Fifty-nine volatile compounds were identified in oyster aroma extract. Among these, 25 were responsible for the overall odour of raw oyster. Four compounds identified in oysters were characterised by fresh and marine odour: 3-(E)-hexen-1-ol, decanal, 2-undecanone and 3,6-(E,Z)-nonadien-1-ol. Some compounds were identified for the first time in oysters: 4-(Z)-heptenal (white boiled fish odour), which comes from n-3 polyunsaturated fatty acid oxidation, and 3-octanol (moss and sulphury odour), 2-nonanol (cucumber odour) and octanoic acid, which arise from n-6 polyunsaturated fatty acid oxidation.
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Keywords: Pacific oyster; vacuum steam distillation; olfactometry; aroma; aromatic precursor

INTRODUCTION

Oysters are a valued food in France. They are consumed raw at the beginning of the meal, especially during Christmas and New Year celebrations. France is the largest producer of oyster *Crassostrea gigas* in Europe.[1] Thus, the study of oysters is of real economic interest to oyster farmers. The analysis of aroma in oysters is a convenient means of checking their quality. The aromatic perception of molluscs indicates to the consumer their state of freshness, necessary for the acceptability of the product. Character-impact compounds in oysters are important for describing their sensory qualities.

Gas chromatography/olfactometry (GC/O) is often used in aroma research. This technique can be used after the study of the odour representativeness of extracts.[2,3] In fact, the study of the volatile compounds is often carried out on extracts and not on the original product from which the volatile compounds are extracted. As a result, it is necessary to ensure that the extracts have sensory characteristics close to those of oyster. Moreover, extracts are either in liquid or in gaseous form. Since in the extract there is no interaction between the food matrix and the flavour, the odour of the extract is thus modified. The food matrix is necessary for the odour perception. It could be interesting to reconstitute it[4–6] to improve the odour authenticity.

As soon as a reliable extraction method was found,

GC/O was applied to extracts to characterise the most potent odorants. The contribution of volatile compounds to the aroma of a food depends on their threshold. Moreover, all humans are not biologically equal in odour threshold. The odour threshold found in the literature was used to explain the potential of a compound as an odorant. This notion is very important for quantitative and descriptive analyses. Even though we are able to detect a large number of volatile compounds, the description of the odour is more difficult.[7]

Josephson et al[8] have found evidence that some volatile compounds in oysters come from certain polyunsaturated fatty acids (PUFAs), well known as aromatic precursors. Our study deals with the identification of the most potent odorants of oysters and aims to correlate the origin of the odorants with the biochemical content of oysters. To date, only Piveteau et al[9] have studied the odorants of oyster extracted by dynamic headspace. A recent study[10] compared two extraction methods: dynamic headspace and vacuum steam distillation. These two methods, using suitably mild conditions, are convenient for raw oysters, which are very sensitive to heat and autoxidation, which could lead to off-flavours. This study, based on the odour authenticity of extracts, showed that vacuum steam distillation gave an extract more like raw oysters than dynamic headspace did.[10]

Our work was aimed at extracting volatile com-

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pounds of oyster *C gigas* and determining the odour-active compounds. The correlation between the sensory profile and the odour-active compounds could thus be made. The origin of some volatiles could be determined by biochemical analyses of the flesh of oysters.

MATERIALS AND METHODS

Reagents
All water used was purified by a Millipore-Q system (Millipore Corp, Saint-Quentin, France). Dichloromethane, 3-hexanone, butylated hydroxytoluene, nonadecanoic acid, trifluoroboride/methanol (14%) and glucose standards were purchased from Sigma-Aldrich (Deisenhofen, Germany). Chloroform was purchased from Cluzeau (Paris, France). Acetone, anhydrous sodium sulphate, sulphuric acid, copper sulphate and sodium potassium tartrate were obtained from Panreac (Madrid, Spain). Hexane, toluene, phenol, trichloroacetic acid, sodium hydroxide, anhydrous sodium carbonate, Folin–Ciocalteu reagent and bovine albumin were purchased from Merck (Darmstadt, Germany).

Oysters
Adult Pacific oysters *C gigas* were obtained from the Bay of Bourgneuf on the Atlantic coast of France. They were fed with phytoplankton contained in seawater at 14°C. They were collected in a marine farm from January to March 2001 (outside the maturation period). Following collection, they were transported under refrigerated conditions and stored at 4°C in our laboratory until analysis. Analyses were performed on live oysters within 6 days. During this time the sensory qualities of oysters were not modified. The whole weight of oyster and the flesh weight were measured on 30 oysters. The condition index (CI) was calculated as CI = $wf \times 100/wt$, where wf = flesh weight and wt = oyster total weight. This index was calculated in order to check how the oyster uses the internal cavity volume for the development of its tissues. It is a convenient method to evaluate the yield of oyster. The condition index of the oysters used was 6.7. This index can vary from 6 to 9 according to the quality of the flesh.

Volatile compounds
Vacuum steam distillation
Vacuum steam distillation was performed using the method of Fords and Holloway.[11] A 200 g portion of whole raw oyster flesh and 1 ml of internal standard (3-hexanone, 10 µg ml⁻¹) were placed in a 6 l flask with 300 ml of water. The flask was placed in a thermostatic water bath at 20°C. It was connected to two condenser columns set at −1°C. The condenser columns were connected to a 4 l flask placed in a thermostatic water bath at 2°C and to three traps cooled with liquid nitrogen at −196°C. The volatile compounds extracted were collected in the 4 l flask

and the three traps. The pressure was maintained at 600 Pa for 4.5 h (time necessary to evaporate 300 ml of ultrapure water and 70% of intrinsic water of oysters). Distillations on 200 g of oysters were carried out in triplicate. After each distillation the contents of the 4 l flask and the three traps were pooled and extracted by 3 × 30 ml of fresh dichloromethane. The organic extract was concentrated to 10 ml at 45°C using a Kuderna-Danish (Batailler, Nantes, France) apparatus consisting of a 100 ml blister equipped with a Snyder (Batailler, Nantes, France) column. Then it was concentrated to 1 ml under a gentle nitrogen stream at room temperature.

Preparation of extract for volatile compound analyses
The three organic extracts thus obtained were pooled and concentrated to 0.5 ml under a nitrogen stream at room temperature. The extract was sealed with a Teflon cap and stored at −20°C prior to use.

Quantification
A gas chromatograph (Star 3400, Varian, Palo Alto, CA, USA) was used. The volatile compounds were separated on a capillary column (DB-Wax, 30 m length × 0.32 mm id × 0.5 µm thickness, J&W Scientific, Folsom, CA, USA) and detected by a flame ionisation detector. The helium carrier gas flow rate was 1 ml min⁻¹. A 3 µl aliquot of vacuum steam distillation extract was injected at 250°C. The detector was set at 250°C. The oven temperature was programmed from 50 up to 70°C at 6°C min⁻¹, followed by an increase to 150°C at 4°C min⁻¹ and then up to 250°C for 10 min at 10°C min⁻¹.

Identification by gas chromatography/mass spectrometry (GC/MS)
The GC/MS system consisted of an HP 5890 II gas chromatograph and an HP 5971 II mass-selective detector (MSD) (Hewlett Packard Co, Palo Alto, CA, USA). A 3 µl aliquot of vacuum steam distillation extract was injected into a capillary column, the same as used for quantification and with the same oven temperature programme. The helium carrier gas flow rate was 1 ml min⁻¹. The MS (electronic impact ionisation) conditions were: ionisation energy, 70 eV; mass range, *m/z* 33–300; scan rate, 2.0 scans s⁻¹; electron multiplier voltage, 200 V; interface temperature, 180°C.

The volatile compounds were identified by matching their spectra with those of a commercial database (NBS 75k) and of an internal library of our laboratory. The retention index of each volatile compound, calculated according to Van Den Dool and Kratz,[12] was compared with the literature. Chemical standards of some volatile compounds were injected directly into the GC/MS system.

Gas chromatography/olfactometry (GC/O)
GC/O involved the smelling of the effluent by ten panellists. Each judge was asked to signal when an
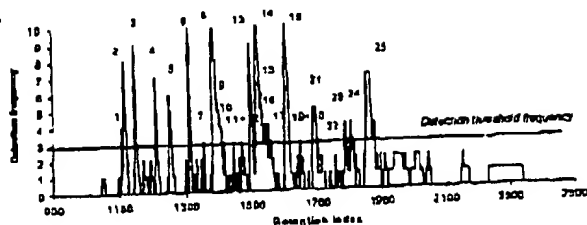
Figure 1. Aromagram of volatile compounds of oyster *Crassostrea gigas* obtained by olfactometry global method. Peak numbers correspond to those of Table 2.

odour was perceptible, to give an odour descriptor[13] and to assess the intensity of the odour on a scale of 1–9 (1=very weak odour intensity, 9=very strong odour intensity). Each judge participated in sniffing for 20 min maximum. Owing to this short period of sniffing, the judges stayed alert. An odour smelled by less than three panellists was considered as noise.[14] The 10 individual aromagrams were summed to yield a final aromagram (detection frequency *versus* retention index) (see Fig 1). The GC/O system consisted of a gas chromatograph (Star 3400, Varian) and a sniffing port supplied with humidified air at 40°C. A 3 µl aliquot of vacuum steam distillation extract was injected in splitless mode at 250°C into a capillary column, the same as used for quantification. The helium carrier gas flow rate was 1 ml min^{-1}. The oven temperature was programmed as for quantification.

Sensory evaluation of oyster and aroma extract

Seven judges trained in seafood aroma recognition generated descriptors for fresh raw oysters.[10] A list of seven descriptors was established after discussion between the panellists to eliminate inappropriate or redundant ones. These descriptors were oyster, seaside, seaweed, grass, cucumber, floral and mud. The descriptor cardboard was added to this list at the judges' request to evaluate the odour of vacuum steam distillation oyster extract. The oyster extract, after application on an odour blotter strip, and the oyster were presented blindly in brown flasks. The panel assessed the intensity of each descriptor for fresh raw oyster and vacuum steam distillation extract on an unstructured scale of 10 cm (0 (left-hand anchor)=no odour intensity, 10 (right-hand anchor)=strong odour intensity). The intensities given by the seven judges for each descriptor were summed to provide a sensory profile of fresh raw oyster and vacuum steam distillation oyster extract (see Fig 2).

Chemical analyses of oyster tissue

Protein, carbohydrate and glycogen contents were measured by spectrophotometric techniques. The protein content of 30 freeze-dried oysters was determined as described by Lowry *et al*.[15] The carbohydrate and glycogen contents of 30 freeze-dried oysters were determined by the method of Dubois *et al*.[16] Lipids were assessed by gravimetric techniques

after solvent extraction, using a chloroform/methanol mixture (2:1 v/v), according to the method of Folch *et al*.[17]

Fatty acid composition

Total lipid extracts were transmethylated with methanol/trifluoroboride (14%) using the method of Morrison and Smith.[18] The fatty acid methyl esters (FAMEs) thus obtained were analysed using a gas chromatograph (HP 5890 II, Hewlett Packard Co) equipped with a flame ionisation detector. A 1 µl aliquot of FAME extract was injected at 250°C and detected at 280°C. The FAMEs were separated on a capillary column (DB-23, 30 m length × 0.25 mm id × 0.25 µm thickness, J&W Scientific). The helium carrier gas flow rate was 1 ml min^{-1}. The oven temperature was programmed from 150°C for 3 min up to 180°C for 7 min at a rate of 10°C min^{-1}, followed by an increase to 215°C for 15 min at a rate of 5°C min^{-1}. The FAMEs were identified by comparing their retention times with those of a standard mixture (Sigma Chemical Co, St Louis, MO, USA). The identification of each FAME was confirmed using a GC/MS system consisting of an HP 5890 II gas chromatograph and an HP 5971 II MSD (Hewlett Packard Co). The GC conditions (oven temperature programme, injector and detector temperatures) were the same as described above. The MSD conditions were: ionisation energy, 70 eV; mass range, m/z 33–300; scan rate, 2.0 scans^{-1}; electron multiplier voltage, 200 V; interface temperature, 180°C. The spectra of FAMEs were compared with those of a commercial database (NBS 75k) and of an internal library of our laboratory.

Statistical treatment

Data acquisition and statistical treatment (ANOVA) were performed with Statgraph 4.0 software (Statistical Graphics Corp, New Jersey, USA).
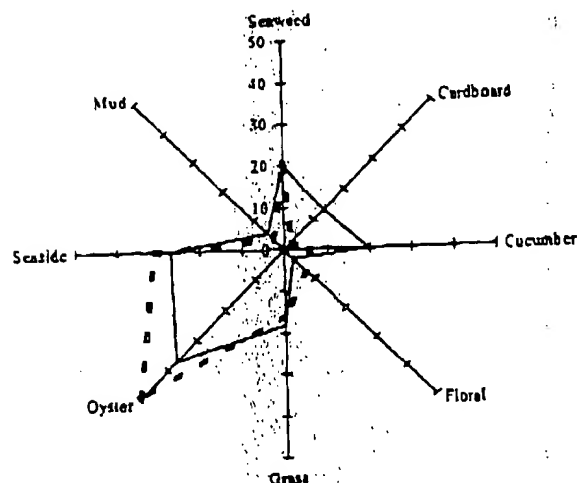

Figure 2. Sensory profile of oyster reference (broken line) and vacuum steam distillation extract (full line).

RESULTS AND DISCUSSION
Identification of volatile compounds by GC/O

The isolation of volatile compounds of oyster *C gigas* was done using vacuum steam distillation. This method provided an extract representative of the original product. The dichloromethane oyster extract of vacuum steam distillation was injected into the GC/MS system. Fifty-nine volatile compounds were identified (Table 1). Alcohols were the major chemical class detected, with 18 volatile compounds. The oyster extract also contained 10 aldehydes, six ketones and eight alkaloids. Josephson *et al*[8] and Piveteau *et al*[9] have studied volatile compounds of oyster *C gigas* extracted by dynamic headspace. Josephson *et al*[8] identified 30 volatile compounds, of which four were in agreement with our findings. These compounds were 2,4-(*E,E*)-heptadienal, 2,6-(*E,Z*)-nonadienal, 1-octen-3-ol and nonanal. Piveteau *et al*[9] tentatively identified 52 volatile compounds, of which 14 were in agreement with our findings. These compounds were 1,3-(*E*)-5-(*Z*)-octatriene, 2-(*E*)-pentenal, 1-penten-3-ol, 2-(*E*)-hexenal, 2-(*E*)-penten-1-ol, 2,4-(*E,E*)-heptadienal, 2,6-(*E,Z*)-nonadienal, 2-(*Z*)-octenal, 1-octen-3-ol, 2-(*E*)-octen-1-ol, octanal, decanal, 3-octanone and 6-methyl-5-hepten-2-one.

Dynamic headspace is a method that extracts low-boiling-point components, whereas vacuum steam distillation extracts medium- and high-boiling-point components. This could explain the relative differences between the volatile compounds identified. Indeed, Josephson *et al*[8] and Piveteau *et al*[9] identified a number of low-boiling-point components which were probably lost during vacuum steam distillation. On the other hand, high-boiling-point components were identified by vacuum steam distillation, whereas they were not extracted and therefore not identified by dynamic headspace. Cha[19] studied raw oyster *Crassostrea virginica* (Atlantic oyster) extracted by simultaneous vacuum distillation/extraction. Seven compounds were the same as in our study. These were 1-penten-3-ol, 2-(*E*)-penten-1-ol, 1-octen-3-ol, octanal, limonene, 2-ethyl-hexan-1-ol and 2-undecanone. Kim *et al*[20] studied an oyster by-product (cooked oyster *C gigas* effluent). They used the same extraction method as Cha.[19] Two compounds were in agreement with our study: 2,6-(*E,Z*)-nonadienal and methional.

Fig 1 shows an aromagram of the odorant compounds identified in the vacuum steam distillation oyster extract. Twenty-five compounds are potent odorants of oysters. The numbered peaks correspond to odours detected by at least three judges. The numbers above the peaks correspond to the numbers in Table 2. Nine compounds can be considered as the most potent odorants of oyster as they were detected by at least seven judges out of 10. All 10 judges smelled 2-(*E*)-penten-1-ol (6) with a mushroom odour, 3-(*E*)-hexen-1-ol (8) with a moss and fresh odour, decanal (14) with a marine odour, which had a high odour detection threshold (10000 ppb),[21] and 2-undecanone

(18) with a cucumber and fresh odour. Nine judges smelled 2-(*E*)-pentenal (3), which had a high odour detection threshold (1500 ppb),[21] and was characterised by a grass odour, and 2,4-(*E,E*)-heptadienal (13), characterised by a mushroom and moss odour. Unknown compound 2 with a sulphury and garlic odour was detected by eight judges. Limonene (4) (odour detection threshold of 10 ppb),[21] characterised by a moss and green odour, and unknown compound 25, characterised by a cucumber and green odour, were detected by seven judges.

Seven compounds were detected by only three judges. These compounds were characterised by heavy odours, ie grilled for ethylpyrazine (7), mushroom for unknown compound 10, boiled potato for 1-octen-3-ol plus methional (11+12), cucumber for 2,6-(*E,Z*)-nonadienal (17) and animal for aceto-phenone plus 3-methylthio-1-propanol (19+20). 3-Methylthio-1-propanol could be a reduction compound of methional.[23] Ethylpyrazine, which had a high odour detection threshold of 6000–22000 ppb,[21] was present in small quantity in oyster extract (0.8 ng equivalents 3-hexanone g^{-1} dry matter of oyster; Table 2). Its high detection threshold could explain why it was detected by only three judges. 1-Octen-3-ol and methional, whose detection thresholds were 1 and 0.2 ppb respectively[21] and whose combined concentration was 346.4 ng equivalents 3-hexanone g^{-1} dry matter of oyster, surprisingly were only detected by three judges. One hypothesis is that these compounds could be provided by co-elution of another non-odorant compound, which could explain the high quantity found and the few judges who detected this odour. 2,6-(*E,Z*)-Nonadienal was detected by only three judges in spite of its low odour detection threshold (0.01 ppb).[21] However, it is present in small amounts in oyster (3.5 ng equivalents 3-hexanone g^{-1} dry matter of oyster; Table 2). Most of the identified dry matter of oyster were characterised by fresh odorant compounds were characterised by fresh odours, eg green (1, 4, 23 and 25), grass (3), fresh (8), marine (14 and 22) and cucumber (15–18). There were also some grilled and cooked odours, eg white boiled fish (5), grilled (7 and 21) and boiled potato (11+12). One hypothesis is that ethylpyrazine (7) and acetylpyrazine (21), responsible for grilled odour, could be generated during the extraction and concentration of the vacuum steam distillation extract.

Among the 25 detected odour-impact compounds, 12 have previously been identified in oyster or an oyster by-product. 2,4-(*E,E*)-Heptadienal with a mushroom and moss odour, 1-octen-3-ol with a boiled potato odour (when associated with methional) and 2,6-(*E,Z*)-nonadienal with a cucumber odour were all identified in dynamic headspace extract of oyster *C gigas*.[8,9] 1-Octen-3-ol was also identified by Cha[19] in raw oyster *C virginica*. Kim *et al*[20] also detected the presence of 2,6-(*E,Z*)-nonadienal in cooked oyster *C gigas* effluent. These last two authors used simultaneous vacuum distillation/extraction to isolate the volatile compounds. Piveteau *et al*[9] identified 1,3-(*E*)-

Table 1. Volatile compounds of oyster *Crassostrea gigas*

Compound	Retention index	Method(s) of identification	Reference(s)[a]	Detection threshold (ppb)
n-3 PUFA oxidation				
1,3-(E)-5-(Z)-Octatriene	1108	RI	9	1500[b]
2-(E)-Pentenal	1124	RI, MS, std	9	400[b]
1-Penten-3-ol	1170	RI, MS	9, 19	17[b]
2-(E)-Hexenal	1221	RI, MS	9	0.8–1[b]
4-(Z)-Heptanal	1236	RI		
2-(E)-Penten-1-ol	1321	RI, MS, std	8, 9	
2,4-(E,E)-Heptadienal	1488	RI, MS	8, 9, 20	0.01[b]
2,6-(E,Z)-Nonadienal	1584	RI, MS, std		
3,5-(E,E)-Octadien-2-one	1570	RI, MS		4000[b]
n-6 PUFA oxidation				
1-Pentanol	1303	RI, MS, std		3[b]
2-(Z)-Octenal	1413	RI, MS	8, 9, 19	1[b]
1-Octen-3-ol	1451	RI, MS, std	9	
2-(E)-Octen-1-ol	1618	RI, MS		
Pentanoic acid	1734	RI, MS		3000[b]
Octanoic acid	1845	RI, MS		0.7[b]
n-9 MUFA oxidation			9, 19	
Octanal	1289	RI, MS		
1-Hepten-3-ol	1351	RI, MS, std		1[b]
Nonanal	1392	RI, MS, std	8	3[b]
Heptanol	1457	RI, MS, std		10000[b]
Decanal	1497	RI, MS, std	9	110–130[b]
1-Octanol	1553	RI, std		
Fatty acid oxidation			9	28[b]
3-Octanone	1256	RI, MS, std		
Carotenoid degradation			9	50[c]
6-Methyl-5-hepten-2-one	1338	RI, MS, std		
Polysaccharide degradation				
Ethylbenzene	1132	RI, MS		
p-Xylene	1140	RI, MS		
1,2,4-Trimethylbenzene	1280	RI, MS		5900[b]
Phenol	1996	RI, MS, std		6000–22000[b]
Maillard reaction				
Ethylpyrazine	1354	RI, std		62[b]
Acetylpyrazine	1647	RI		
Strecker reaction			20	0.2[b]
Methional	1451	RI, std		
Unknown origin				1[b]
Dimethyl sulphide	773	RI, MS, std		
3-Penten-2-ol	1181	RI, MS, std		
Dodecane	1200	RI, MS, std	18	10[b]
Limonene	1201	RI, MS, std		
3-Hexanol	1211	RI, MS		
1-Dodecene	1236	RI, MS, std		
Tridecane	1290	RI, MS, std		
3-Nonanone	1357	RI, MS		
2,4,6-Trimethylpyridine	1378	RI, MS, std		
3-(E)-Hexen-1-ol	1386	RI, MS, std		
Tetradecane	1390	RI, MS		
3-Octanol	1395	RI, MS		
2-Butoxyethan-1-ol	1406	RI, MS, std	19	
7-(Z)-Tetradecene	1465	RI, MS, std		
2-Ethyl-hexan-1-ol	1490	RI, MS, std		
2-Nonanol	1535	RI, MS, std	18	
Hexadecane	1590	RI, MS, std		
2-Undecanone	1596	RI, MS		500000[b]
Hexadecene	1630	RI, MS, std		
Acetophenone	1645	RI		
3-Methylthio-1-propanol	1645	RI, MS, std		
2,6,10,14-Tetramethylpentadecane	1655	RI, MS, std		
Heptadecane	1688	RI, MS, std		

Table 1. Continued

Compound	Retention Index	Method(s) of identification	Reference(s)[a]	Detection threshold (ppb)
Unknown origin				
3,6-(E,Z)-Nonadien-1-ol	1731	RI, MS		
Octadecane	1789	RI, MS, std		
Octadecane	1823	RI, MS		
Nonadecane	1889	RI, MS		
Butylated hydroxytoluene	1902	RI, MS, std		
Eicosane	1966	RI, MS, std		

RI, retention index on DB-Wax capillary column; MS, mass spectrometry; std, chemical standard.

[a] Previously reported in the literature as a volatile compound in oyster.

[b] From Ref 21.

[c] From Ref 22.

5-(Z)-octatriene (plastic and green odour), 2-(E)-pentenal (grass odour), 2-(E)-penten-1-ol (mushroom odour) and decanal (marine odour) in C gigas. 2-(E)-Penten-1-ol was also identified by Cha.[19] This last author also identified limonene (moss and green odour) and 2-undecanone (cucumber and fresh odour). Methional with a boiled potato odour (when associated with 1-octen-3-ol) was identified in cooked oyster effluent.[20] 1,3-(E)-5-(Z)-Octatriene, 2-(E)-pentenal and 2,4-(E,E)-heptadienal were only found in oyster C gigas, whereas limonene, 2-ethyl-1-hexanol and 2-undecanone were previously detected in oyster C virginica. Methional has only been identified in cooked oyster effluent. Its formation could be due to

heating during the preparation of the sample or the extraction of the volatile compounds.

Some volatile compounds characteristic of green and fresh odour were identified by dynamic headspace as well as by vacuum steam distillation. Indeed, 1,3-(E)-5-(Z)-octatriene, 2-(E)-pentenal and decanal were found in oyster extract using these two isolation methods. This could be explained by the suitably mild conditions used, particularly little heating. Four of these compounds were unknown and had sulphury and garlic (2), mushroom (10), green (23) and cucumber and green (25) odours. Unknown compound 2 could not be identified because it was co-eluted with the solvent. Unknown compound 10 was

Table 2. Odour-active compounds of oyster Crassostrea gigas

Peak	Retention index	Compound[a]	Methods of identification	Odour descriptor[b]	Detection frequency[c]	Average Intensity	Quantity[d]
1	1108	1,3-(E)-5-(Z)-Octatriene	RI, odour	Plastic, green	4	2.1	72.9
2	1118	Unknown		Sulphury, garlic	8	3.8	2.1
3	1124	2-(E)-Pentenal (1500)	RI, MS, odour, std	Grass	9	4.0	1.3
4	1201	Limonene (10)	RI, MS, odour, std	Moss, green	7	2.8	6.4
5	1236	4-(Z)-Heptenal	RI, odour	White boiled fish	6	2.8	5.3
6	1321	2-(E)-Penten-1-ol	RI, MS, odour, std	Mushroom	10	7.4	19.3
7	1354	Ethylpyrazine (6000–22000)	RI, odour, std	Grilled	3	1.2	0.8
8	1386	3-(E)-Hexen-1-ol	RI, MS, odour, std	Moss, fresh	10	7.9	9.5
9	1395	3-Octanol	RI, MS, odour	Moss, sulphury	6	4.2	Trace
10	1417	Unknown		Mushroom	3	1.1	9.9
11+12	1451	1-Octen-3-ol (1) + methional (0.2)	RI, MS, odour, std	Boiled potato	3	1.4	346.4
13	1486	2,4-(E,E)-Heptadienal	RI, MS, odour	Mushroom, moss	9	5.3	0.8
14	1497	Decanal (10000)	RI, MS, odour, std	Marine	10	5.8	15.8
15	1535	2-Nonanol	RI, MS, odour, std	Cucumber	6	3.8	2.0
16	1553	1-Octanol (110–130)	RI, odour, std	Cucumber	4	2.3	1.5
17	1584	2,6-(E,Z)-Nonadienal (0.01)	RI, MS, odour, std	Cucumber	3	1.7	3.5
18	1596	2-Undecanone	RI, MS, odour, std	Cucumber, fresh	10	5.9	47.8
19+20	1645	Acetophenone + 3-methylthio-1-propanol	RI, MS, odour, std	Animal	9	0.9	0.3
21	1647	Acetylpyrazine	RI, odour	Grilled	6	2.7	2.6
22	1731	3,6-(E,Z)-Nonadien-1-ol	RI, MS, odour	Marine	4	1.1	90.8
23	1806	Unknown		Green	4	1.8	27.1
24	1845	Octanoic acid (3000)	RI, MS, odour	NC	4	2.5	9.9
25	1858	Unknown		Cucumber, green	7	3.3	44.1

RI, retention index on DB-Wax capillary column; MS, mass spectrometry; std, chemical standard; NC, non-common descriptor.

[a] Detection threshold (ppb) in parentheses.

[b] As given by olfactometry.

[c] Number of judges out of 10 who detected the odour.

[d] Given as ng equivalents 3-hexanone g^{-1} dry matter of oyster.

probably not in the MS libraries. Unknown compounds 23 and 25 were confounded with the MS background owing to the increase in GC temperature.

Nine other odour-impact compounds were identified in raw oyster *C gigas* for the first time. 2-Nonanol and acetophenone plus 3-methylthio-1-propanol were identified by their retention index and mass spectrometry, and this was confirmed by their odour and the injection of corresponding standards. 3-Octanol and octanoic acid were identified by their retention index, mass spectrometry and their odour. Ethylpyrazine and 1-octanol were identified by their retention index and odour, and this was confirmed by their standard. 4-(*Z*)-Heptenal and acetylpyrazine were identified by their retention index and odour. Three of these compounds were detected by six judges. These were 4-(*Z*)-heptenal with a white boiled fish odour, 3-octanol with a moss and sulphury odour and 2-nonanol with a cucumber odour. 1-Octanol, characterised by a cucumber odour, was detected by four judges. Acetophenone plus 3-methylthio-1-propanol, characterised by an animal odour, and octanoic acid (odorant, but no common descriptor was found for it) were smelled by three and four judges respectively. Ethylpyrazine and acetylpyrazine with a grilled odour were detected by three and five judges respectively. The last two compounds were not characteristic of fresh odour (and thus of raw oyster). They were probably due to exposure to heat during the different extraction steps of the volatile compounds of oysters.

Among the detected odour-impact compounds, some have been identified in other seafoods. For instance, 4-(*Z*)-heptenal, ethylpyrazine, methional and acetylpyrazine were also detected in cooked mussels.[2] 2-(*E*)-Pentenal, 2-(*E*)-penten-1-ol, 1-octen-3-ol, 2,4-(*E,E*)-heptadienal, 2,6-(*E,Z*)-nonadienal and 2-undecanone were also identified in crayfish waste.[24] 2-(*E*)-Penten-1-ol and 2,4-(*E,E*)-heptadienal, both with a mushroom odour, were found in a marine green alga (*Ulva pertusa*)[25] and in ayu fish.[26] Alga *U pertusa* and ayu fish also contained 1-octen-3-ol and 2,6-(*E,Z*)-nonadienal, both with a cucumber odour.[25,26]

Correlation between sensory profile and odour-active compounds

It is interesting to note a good correlation between the sensory profile of oyster *C gigas*, the sensory profile of oyster aroma extracted after vacuum steam distillation and the odour-active compounds of oyster (Fig 2 and Table 3). Oyster was described by odours such as oyster, seaside, seaweed and grass. Vacuum steam distillation extract of oyster was described by the same descriptors plus cucumber and cardboard. Cardboard odour is probably a taint caused by oxidation of oyster. Harayama *et al*[27] reported that cardboard odour could be formed by 2-(*E*)-nonenal, but also by other carbonyls which have the same odour. These compounds were not identified in our extract, perhaps because they were masked by other compounds or

because they were in trace concentration. Another hypothesis is that the use of odour blotter strips for sensory evaluation could provoke the taint of cardboard odour. The descriptors floral and mud were found in low amounts for both raw oyster and vacuum steam distillation oyster extract. No odour characterising these descriptors was found in vacuum steam distillation oyster extract.

Four compounds of oyster extract were responsible for fresh and marine odours, which could be correlated with oyster, seaside and seaweed descriptors of the sensory profile. These four compounds were 3-(*E*)-hexen-1-ol and 2-undecanone for fresh odour and decanal and 3,6-(*E,Z*)-nonadienol for marine odour. Their detection frequencies were respectively 10, 10, 10 and four out of 10. These compounds contributed to the overall odour of raw oyster. Five compounds of oyster extract had a green odour, which could be correlated with the grass descriptor. 1,3-(*E*)-5-(*Z*)-Octatriene, 2-(*E*)-pentenal, limonene and unknown compounds 23 and 25 had detection frequencies of four, nine, seven, four and seven out of 10 respectively. They contributed greatly to the odour of oyster extract.

Fig 2 shows that raw oyster and oyster steam distillation extract had about the same sensory characteristics for descriptors oyster, seaside, seaweed and grass. As a result, the nine compounds previously cited contributed either to oyster extract or to raw oyster. As for the cucumber descriptor and odour, five compounds characterised it: 2-nonanol, 1-octanol, 2,6-(*E,Z*)-nonadienal, 2-undecanone and unknown compound 25. These compounds had detection frequencies of six, four, three, 10 and seven out of 10 respectively, which related to a high contribution of these compounds in oyster extract. Fig 2 shows a high

Table 3. Correlation between sensory profile and odorants of vacuum steam distillation extract

Sensory descriptor[a]	Odour descriptor[b]	Odorants[c]
Oyster Seaside Seaweed	Fresh	3-(*E*)-Hexen-1-ol (10) 2-Undecanone (10) Decanal (10)
	Marine	3,6-(*E,Z*)-Nonadien-1-ol (4)
Grass	Green	1,3-(*E*)-5-(*Z*)-Octatriene (4) 2-(*E*)-Pentenal (9) Limonene (7) Unknown 23 (4) Unknown 25 (7)
Cucumber	Cucumber	2-Nonanol (6) 1-Octanol (4) 2,6-(*E,Z*)-Nonadienal (3) 2-Undecanone (10) Unknown 25 (7)

[a] Sensory analysis of oyster extract.
[b] Odour descriptor found by olfactometric method in oyster extract.
[c] In parentheses, number of judges out of 10 who smelled the odour in oyster extract (the same as the detection frequency in Table 2).

contribution of cucumber odour in the extract, whereas this descriptor was very weak in raw oyster. This odour was produced during the extraction and was probably caused by fatty acid oxidation. That is why it was found mostly in the extract.

Aromatic precursors

Oysters *C gigas* are composed mainly of protein and ash (respectively 49.9 and 25.0% of dry matter). They also contain lipids (8.5% of dry matter), carbohydrates and glycogen. Among lipids, 53.0% of total fatty acids are polyunsaturated fatty acids (PUFAs). The main PUFAs are n-3 PUFAs (46.3% of total fatty acids), mostly C20:5n-3 and C22:6n-3 (respectively 23.5 and 21.7% of total fatty acids). However, there are also non-negligible amounts of n-6 PUFAs (7.3% of total fatty acids) and n-9 monounsaturated fatty acids (6.2% of total fatty acids). PUFAs are very sensitive to oxidation because of their high level of unsaturation. They can give rise to the formation of carbonyl compounds during oxidation reactions. Twenty-one out of the 59 compounds arose from the oxidation of fatty acids. Among them, 10 were odour-active compounds probably resulting from the degradation of n-3, n-6 and n-9 fatty acids.

The most odour-active compounds came from the oxidation of n-3 PUFAs (six out of 10). 2,6-(*E*,*Z*)-Nonadienal (cucumber odour) could arise from the oxidation of C20:5n-3[28] and C18:3n-3[29] (1.0% of total fatty acids). 4-(*Z*)-Heptenal could result from the oxidation of n-3 PUFAs.[30] This compound, characterised by a white boiled fish odour, could also be generated by oxidative degradation of 2,6-(*E*,*Z*)-nonadienal.[14] 2-(*E*)-Pentenal, characterised by a grass odour, could come from the oxidation of n-3 PUFAs via 15-lipoxygenase[14] or, more precisely, from the oxidative degradation of linolenic acid.[31] 1,3-(*E*)-5-(*Z*)-Octatriene, characterised by a plastic and green odour, and 2-(*E*)-penten-1-ol, characterised by a mushroom odour, could arise from the oxidative degradation of n-3 PUFAs,[9] but their precise origin is unknown. 2,4-(*E*,*E*)-Heptadienal, characterised by a mushroom and moss odour, could result from the oxidation of linolenic acid.[30] 1-Octen-3-ol, which was eluted together with methional and was characterised by a boiled potato odour, and octanoic acid, whose odour could not be described by the judges, came from n-6 PUFA degradation.[30] Two odorant compounds could come from the oxidative degradation of n-9 fatty acids. Decanal with a marine odour could arise from the oxidative degradation of C18:1n-9[30] (2.1% of total fatty acids). 1-Octanol with a cucumber odour could also come from the oxidation of C18:1n-9.[31]

Other odorants have known origins other than fatty acid oxidation. Methional, which had a low odour detection threshold and was characterised by a boiled potato odour, could be provided by the degradation of methionine by way of the Strecker reaction.[14] Methionine could be formed from carbonyl compounds generated during the oxidation of fatty acids.[14]

Ethylpyrazine and acetylpyrazine, both characterised by a grilled odour, could be generated by the Maillard reaction.[32]

In addition to the 13 odorants whose origin was known, some of the 59 identified compounds have a known origin (Table 1). Among them, 2-(*Z*)-octenal could be provided by oxidation from both linoleic acid and arachidonic acid,[30] which comprise respectively 0.6 and 4.3% of total fatty acids. Octanal and nonanal could arise from the oxidative degradation of C18:1n-9[30] (2.1% of total fatty acids). Four volatile compounds (ethylbenzene, *p*-xylene, 1,2,4-trimethyl-benzene and phenol) could come from glucose degradation.[33] Finally, it is interesting to note the presence of 6-methyl-5-hepten-2-one, which may be generated by carotenoid degradation.[9]

CONCLUSION

Vacuum steam distillation has enabled 59 volatile compounds to be identified in oyster *C gigas*, among which 25 are odour-active compounds. Many of the odour-active compounds had a fresh, marine and green odour that is very characteristic of the raw product. The contribution of the cucumber odour was characteristic in the oyster extract. This odour was present in low amount in raw oyster. It was generated by the extraction method and by fatty acid oxidation.

This study shows that the composition of volatile compounds in oyster *C gigas* depends on the biochemical composition and essentially on the composition of fatty acids. Indeed, 13 odour-active compounds had a known origin. Among these, 10 came from the oxidative degradation of fatty acids.

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Composition of the essential oil of *Pinus canariensis* Sweet ex Sprengel

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ABSTRACT: The essential oil of *Pinus canariensis* Sweet ex Sprengel obtained by steam distillation of needles was investigated by GC and GC–MS. One hundred and sixteen components were separated, of which 75 (comprising 93.9% w/w of the sample) were positively identified and a further 41 partially characterized. One hundred and eight substances could be assigned to terpenoids: 33 monoterpenes (42.7%), 46 sesquiterpenes (52.1%), and 29 diterpenes (4.8%). Minor substances were alkane derivatives as well as the benzyl esters of benzoic and salicylic acid. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS: *Pinus canariensis* Sweet ex Sprengel; Pinaceae; essential oil composition; hydrodistillation; GC; GC–MS; monoterpenes; sesquiterpenes; diterpenes

Introduction

The genus *Pinus* (Pinaceae) comprises more than 100 species and is widespread in the northern hemisphere. *Pinus canariensis* Sweet ex Sprengel is an endemic tree of the Canary archipelago whose natural distribution area is restricted to the highest islands. In Tenerife, it grows spontaneously from near sea level up to about 2200 m. In Italy, and to a smaller extent elsewhere in the Mediterranean region, *P. canariensis* is planted for timber.

The chemical composition of various pine species volatiles have been the subject of numerous studies.[1-6] The majority of the studies focused on North American and Central European species and only a limited number of chemically oriented reports dealt with Mediterranean pine species.[4,6] Very little is known about the chemical composition of the volatile metabolites of *Pinus canariensis*. One study dealt with the composition of the essential oil of *P. canariensis* growing in Greece.[5] This paper presents results of a GC and GC–MS investigation of *P. canariensis* from the natural habitat in Tenerife, Canary Islands.

Experimental

Plant Material and Essential Oil Isolation

Branches of 20 *Pinus canariensis* trees were collected at Las Raices near La Esperanza, Tenerife (latitude 28°25′N,16°23′W, elevation 1100 m). The trees were 20–60 years old. Needles were taken in February 1999 from fully developed one-year-old shoots. 12–41 g of pine needles were cut into small pieces and steam distilled in a Karlsruher-type apparatus[7] for 6 h. Volatiles were collected into 1.0 ml *n*-pentane. The oils were dried over anhydrous sodium sulphate and stored at −20°C in a refrigerator.

Gas Chromatography (GC) and GC–Mass Spectrometry Analysis

GC profiles were established on a DANI 8610 and a DANI 8400 Capillary Gas Chromatograph each equipped with a Programmed Temperature Vaporizer (PTV) injection system, a flame ionization detector (FID) and a LDC/Milton Roy C1-10 B integrator. The samples were analyzed on fused silica capillary columns with bonded phases of different polarity.

The non-polar system comprised a CP-Sil 5 CB (50 m × 0.22 mm i.d.; film thickness 0.13 μm) capillary column. Hydrogen was the carrier gas with a linear velocity of 43 cm/s. Column temperature programming was: 40–300°C at 4°C/min and 300°C isothermally for 10 min. PTV temperature was 50°C during injection, followed by a very rapid heating to 280°C. The FID was operated at 310°C.

The polar system included a DB-Wax (60 m × 0.32 mm i.d.; film thickness 0.25 μm) capillary column. Carrier gas (hydrogen) velocity was 53 cm/s. Column temperature programming was: 40°C held for 5 min, and then from 40°C heated at 2.5°C/min to 250°C. PTV temperature was 50°C during injection, followed by a

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very rapid heating to 250°C. The FID was operated at 260°C.

GC–MS was performed on a Hewlett Packard G1800A GCD system (Electron impact voltage: 70 eV, interface temperature 320°C, mass range 30–425 amu). The samples were analyzed on a DB-1 (50 m × 0.20 mm i.d.; film thickness 0.33 μm) capillary column. Other chromatographic conditions were: Carrier gas (helium) at 1 ml/min. Column temperature programming: 40°C initial temperature held for 5 min, then from 40°C heated at 3°C/min to 230°C, and from 230 to 320°C at 10°C/min.

Compounds were identified using both chromatographic and mass spectroscopic criteria. The WILEY275 Database was used for automatic identification of GC–MS peaks. Additionally, linear retention indices[8] obtained on a polar and an apolar column were compared with published data.[9] Mass spectra and retention indices were also compared with data obtained from authentic compounds. Quantitation was achieved from GC–FID profiles on an apolar column according to the area percent method without consideration of calibration factors (F), i.e. $F = 1.0$ for all compounds.

Statistical Analysis

The relationships between the different trees were studied by cluster analysis ('Statistica' software package, StatSoft, USA, 1994).

Results and Discussion

The volatile fraction obtained by hydrodistillation amounted to 0.3% of fresh pine needles. Quantitative data given in Table 1 were obtained from GC–FID profiles using a 50 m fused silica column CP Sil 5 CB. Overall, 116 components were regularly detected as volatiles of *Pinus canariensis* needles, of which 75 (representing 93.9%) were unambiguously identified. The remaining 41 compounds representing 6.1% of the sample were partially characterized on the basis of their mass spectral data and their retention indices.

As in other conifer trees,[3] the majority of *P. canariensis* volatile needle metabolites were terpenoids: 108 substances could be assigned to terpenoids, amounting to 99.6% altogether. Main constituents of the 33 monoterpenes (42.7%) were the hydrocarbons α-pinene (23.1%), β-pinene (1.6%), myrcene (5.8%), and limonene (10.1%). Sesquiterpenes (52.1%) comprised 25 fully identified and further 21 partially characterized compounds with *trans*-caryophyllene (4.9%), α-humulene (1.1%) germacrene-D (35.7%), α-muurolene (1.0%), δ-cadinene (1.4%) and *epi*-α-muurolol (1.1%) as main constituents with more than 1% of the oil. In addition to the mono- and sesquiterpenes, 29 diterpenes could be detected and quantified. Except one oxygenated diterpene ($C_{20}H_{30}O$, RI 2228) which amounted to 1.5%, diterpenes were minor components (together 4.8%). Further minor substances were alkane derivatives as well as the benzyl esters of benzoic and salicylic acid. The highest concentration of a non-terpenoid compound in the needle essential oil of *P. canariensis* needles had *trans*-2-hexenal (0.2%).

In a previous study, 42 substances were detected in the essential oil of *P. canariensis* growing in Greece.[5] Although the number of identified compounds was less than half as compared to this study, the qualitative pattern of monoterpenes was nearly identical. Eighteen of the 22 monoterpenes found in *P. canariensis* growing in Greece were also constituents of the samples from Tenerife. Additional monoterpenes identified in the present study were minor constituents. Less coincidence can be found comparing the patterns of sesqui- and diterpenes. Twenty of these metabolites were detected in essential oils of Greek origin and 15 of them were positively identified.[5] Five main sesquiterpenes appeared also in essential oils from Tenerife, whereas 10 minor constituents did not. On the other hand, 32 minor sesqui- and diterpenes detected in samples from Tenerife were not mentioned in the previous study on Canary Islands pine planted in Greece.

In the present study, the concentration of some compounds varied considerably, which is reflected by a high standard deviation (Table I). This could be due to genetically determined infraspecific chemical variability, as it is well known for essential oils of other pine species, e.g. *Pinus sylvestris*.[1,10,11] Cluster analysis revealed two main groups of trees within the plot, referred to as group 1 and 2. Differences were mainly caused by variation of the content of four monoterpene hydrocarbons: Averagely, the content of myrcene was 4.3-fold and that of limonene was 4.9-fold in trees of group 1 as compared to those of group 2. On the other hand, trees of group 2 had a 3.1-fold content of α-pinene and a 2.6-fold content of β-pinene as compared to the mean values of group 1. There were no striking topological or morphoanatomical differences between samples of the two groups. However, it must be emphasized that the study into the chemistry was not parallelled by a detailed anatomical study. Considering the quantitative results, the pattern of main constituents was in group 1: germacrene D > limonene > α-pinene > myrcene > *trans*-caryophyllene. In group 2, the order of main compounds was: α-pinene > germacrene D > *trans*-caryophyllene > limonene > β-pinene. The most abundant compounds of needle volatiles of *P. canariensis* planted in Greece[5] were: germacrene D > α-pinene > myrcene > δ-cadinene > β-pinene. Their quantitative pattern of terpenoids is similar to trees of Tenerife, but it does not match completely either with

Table 1. Constituents of the essential oil of *Pinus canariensis* Sweet ex Sprengel (column: 50 m CP Sil 5 CB). Partially characterized substances that appeared in traces are not listed

Compound	R.I.	Mean ± S.D. n = 20		Group 1 Mean ± S.D. n = 13		Group 2 Mean ± S.D. n = 7	
trans-2-hexenal	821	0.2	0.06	0.2	0.04	0.2	0.08
cis-3-Hexenol	832	0.1	0.10	0.1	0.04	0.1	0.07
Tricyclene	923	tr	–	tr	–	0.1	0.03
α-Pinene	936	23.1	15.80	13.2	4.09	41.5	12.17
α-Fenchene	945	tr	–	tr	–	tr	–
Camphene	947	0.3	0.22	0.2	0.05	0.5	0.22
Sabinene	968	tr	–	tr	–	tr	–
β-Pinene	974	1.6	1.41	1.0	0.43	2.8	1.92
Myrcene	984	5.8	4.68	7.9	4.38	1.8	1.79
α-Phellandrene	999	tr	–	tr	–	tr	–
Isocineole	1006	tr	–	tr	–	tr	–
α-Terpinene	1011	tr	–	tr	–	tr	–
p-Cymene	1014	tr	–	tr	–	tr	–
β-Phellandrene[a]	1026	0.7	0.34	0.7	0.36	0.7	0.34
Limonene[a]	1026	10.1	10.29	13.9	10.6	2.9	4.83
cis-β-Ocimene	1028	tr	–	tr	–	tr	–
trans-β-Ocimene	1038	0.1	0.14	0.1	0.16	0.1	0.02
γ-Terpinene	1051	tr	–	tr	–	tr	–
Dehydro-*p*-cymene	1073	tr	–	tr	–	0.2	0.09
α-Terpinolene	1081	0.2	0.07	0.1	0.03	0.2	–
Linalool	1084	tr	–	tr	–	tr	–
endo-Fenchol	1101	tr	–	tr	–	tr	–
α-Campholenal	1107	tr	–	tr	–	tr	–
Camphor	1133	tr	–	tr	–	tr	–
Pinocarvone	1143	tr	–	tr	–	tr	–
Borneol	1155	tr	–	tr	–	tr	–
Terpinen-4-ol	1166	tr	–	tr	–	0.4	0.26
α-Terpineol	1176	0.2	0.20	0.1	0.05	tr	–
Myrtenol	1182	tr	–	tr	–	tr	–
Linalyl acetate	1238	tr	–	tr	–	tr	–
Bornyl acetate	1274	0.4	0.18	0.3	0.18	0.5	0.10
α-Terpinyl acetate	1335	0.1	0.14	0.1	0.16	0.1	0.08
α-Cubebene	1354	tr	–	0.1	0.02	tr	–
α-Ylangene	1379	tr	–	tr	–	0.1	0.04
α-Copaene	1383	0.1	0.05	0.2	0.05	0.2	0.06
β-Bourbonene[b]	1391	0.2	0.05	0.2	0.05	0.1	0.06
β-Elemene[a]	1391	0.2	0.06	0.2	0.06	tr	–
Longifolene	1418	tr	–	0.1	0.77	0.4	0.57
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1426	0.6	0.70	5.0	1.90	4.7	1.33
trans-Caryophyllene	1430	4.9	1.69	0.7	0.15	0.5	0.19
β-Cubebene	1436	0.6	0.18	0.3	0.06	0.2	0.07
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1450	0.3	0.07	0.2	0.19	0.1	0.15
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1459	0.2	0.17	1.1	0.37	1.0	0.33
α-Humulene	1461	1.1	0.35	0.3	0.06	0.2	0.06
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1468	0.2	0.07	0.1	0.09	0.1	0.07
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1475	0.1	0.09	0.5	0.69	0.5	0.56
γ-Muurolene	1481	0.5	0.63	39.2	7.43	29.3	10.3
Germacrene D	1493	35.7	9.58	0.5	0.38	0.5	0.23
α-Farnesene	1496	0.5	0.33	tr	–	0.1	0.08
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1498	tr	–	1.1	0.31	0.7	0.27
α-Muurolene	1501	1.0	0.34	0.2	0.16	0.2	0.09
Sesquiterpene hydrocarbon (C$_{15}$H$_{24}$)	1506	0.2	0.14	0.3	0.21	0.2	0.06
β-Cadinene	1508	0.2	0.18	0.6	0.24	0.4	0.15
γ-Cadinene	1516	0.5	0.23	1.6	0.67	1.1	0.41
δ-Cadinene	1522	1.4	0.63	0.1	0.04	tr	–
Epizonarene	1526	0.1	0.04	0.1	0.03	tr	–
4,10-Dimethyl-7-isopropyl-bicyclo[4.4.0]-1,4-decadiene	1533	0.1	0.03	0.1	0.09	0.1	0.07
cis-α-Bisabolene	1536	0.1	0.08	0.1	0.05	0.1	0.02
α-Cadinene	1539	0.1	0.04	0.1	–	0.1	0.12
Dodecanoic acid	1543	tr	–	0.1	0.03	0.1	0.06
Oxygenated sesquiterpene (C$_{15}$H$_{24}$O)	1549	0.1	0.04	0.3	0.29	0.1	0.07
1-*endo*-Bourbonanol	1575	0.3	0.25	0.1	0.04	0.1	0.04
Caryophyllene oxide	1583	0.1	0.04	0.1	0.03	0.1	0.04
Oxygenated sesquiterpene (C$_{15}$H$_{24}$O)	1591	0.1	0.03	0.1	0.04	0.1	0.07
Oxygenated sesquiterpene (C$_{15}$H$_{24}$O)	1605	0.1	0.05	0.2	0.07	0.2	0.14
Oxygenated sesquiterpene (C$_{15}$H$_{24}$O)	1623	0.2	0.10	0.1	0.04	tr	–
Oxygenated sesquiterpene (C$_{15}$H$_{24}$O)	1626	0.1	0.04				

Table 1. Continued

Compound	R.I.	Mean±S.D. n = 20		Group 1 Mean±S.D. n = 13		Group 2 Mean±S.D. n = 7	
α-Cadinol	1636	0.8	0.44	0.9	0.50	0.7	0.26
Oxygenated sesquiterpene ($C_{15}H_{24}O$)	1642	0.1	0.04	0.1	0.04	0.1	0.05
epi-α-Muurolol	1649	1.1	0.62	1.2	0.70	0.9	0.43
1-Tetradecanol	1661	tr	–	tr	–	tr	–
Oxygenated sesquiterpene ($C_{15}H_{26}O$)	1669	0.1	0.04	0.1	0.03	0.1	0.05
Oxygenated sesquiterpene ($C_{15}H_{24}O$)	1675	0.1	0.04	0.1	0.02	0.1	0.05
Oxygenated sesquiterpene ($C_{15}H_{24}O$)	1679	0.2	0.08	0.2	0.05	0.2	0.12
(E,E)-Farnesol	1702	0.1	0.12	0.1	0.06	0.1	0.19
Benzyl benzoate	1733	tr	–	tr		tr	–
Octadecane	1798	tr	–	tr		tr	–
Benzyl salicylate	1841	tr	–	tr		tr	–
1-Hexadecanol	1864	tr	–	tr		tr	–
Diterpene hydrocarbon ($C_{20}H_{33}$)	1959	0.1	0.19	0.1	0.23	0.1	0.08
Sandaracopimaradiene	1967	tr	–	tr		tr	–
Isopimaradiene	2013	0.3	0.17	0.3	0.13	0.2	0.22
13-Epimanoyl oxide	2030	0.1	0.26	0.1	0.32	tr	–
Diterpene hydrocarbon ($C_{20}H_{33}$)	2034	tr	–	tr		0.1	0.05
Diterpene hydrocarbon ($C_{20}H_{32}$)	2045	tr	–	0.1	0.17	tr	–
Diterpene hydrocarbon ($C_{20}H_{32}$)	2096	0.2	0.11	0.2	0.10	0.2	0.12
Phytol	2102	tr	–	tr		tr	–
13(16),14-Labdien-8-ol	2107	0.1	0.22	tr		0.1	0.37
Diterpene hydrocarbon ($C_{20}H_{32}$)	2162	0.1	0.04	0.1	0.02	0.1	0.06
Oxygenated diterpene ($C_{20}H_{30}O$)	2173	0.3	0.22	0.3	0.10	0.3	0.37
Oxygenated diterpene ($C_{20}H_{30}O$)	2228	1.5	0.97	1.6	0.86	1.1	1.14
Oxygenated diterpene ($C_{20}H_{30}O$)	2233	0.1	0.07	0.1	0.06	0.1	0.08
Oxygenated diterpene ($C_{20}H_{34}O$)	2237	0.2	0.10	0.2	0.08	0.2	0.15
Methyl pimarate	2243	tr	–	tr		tr	–
Methyl sandaracopimarate	2260	0.1	0.08	0.1	0.04	0.1	0.12
Methyl isopimarate	2297	0.2	0.10	0.2	0.08	0.2	0.14
Oxygenated diterpene ($C_{21}H_{32}O_2$)	2301	0.1	0.05	0.1	0.06	0.1	0.02
Methyl levopimarate	2307	0.3	0.17	0.4	0.17	0.3	0.18
Oxygenated diterpene ($C_{20}H_{32}O$)	2313	0.3	0.28	0.4	0.28	0.2	0.26
Methyl dehydroabietate	2324	tr	–	tr		tr	–
Oxygenated diterpene ($C_{20}H_{30}O$)	2370	0.5	0.26	0.5	0.29	0.4	0.20
Methyl abietate	2380	0.1	0.07	0.1	0.07	0.1	0.05
Methyl neoabietate	2439	0.2	0.10	0.2	0.10	0.2	0.09
Oxygenated diterpene ($C_{21}H_{30}O_2$)	2468	0.1	0.04	0.1	0.04	0.1	0.04

tr=(<0.05%).
* Percentage on 60 m DB-wax column.

one of both groups, or with the average of all trees investigated in this study. Genetic variability or different environmental influence might be the cause of these differences.

The terpenes are synthesized and accumulated in various types of secretory structures, such as glandular trichomes and resin ducts; secretory ducts are the accumulation sites of the essential oil of pines.[12] To a minor extent, diterpene acids were also detected as constituents of soluble epicuticular lipids.[13] Dodecanoic acid, 1-tetradecanol, octadecane, and 1-hexadecanol are concluded to be constituents of epicuticular lipids of pine needles.[14,15] Long chain alkanes and their derivates usually occur in leaf oils obtained by hydrodistillation in different yield depending on the duration of the distillation.[16]

trans-2-Hexenal and cis-3-hexenol are formed when leaves are damaged.[17] A possible cause for their occurrence in the needle oil of P. canariensis might be that the needles were cut into small pieces before distillation.

On the other hand, strikingly high amounts of trans-2-hexenal were also detected in distillates of oak leaves,[18–20] although entire leaves were processed. Thus, it remains uncertain whether both substances are genuine compounds in pine needles.

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